Expression level of *CRKL* and *AXL* combined with exon 19 deletion in *EGFR* and *ALK* status confer differential prognosis of lung adenocarcinoma subtypes

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Abstract. Non-small cell lung cancer (NSCLC) is a lethal cancer-related disease in population. Adenocarcinoma (AC) is subclassified into several subtypes based on the new classification by the International Association for the Study of Lung Cancer, American Thoracic Society and European Respiratory Society in 2011. Correlation between original expression of Crk-like (*CRKL*) and anaplastic lymphoma receptor tyrosine kinase in diverse histological components of AC and epidermal growth factor receptor (*EGFR*) or *ALK* status was evaluated by immunohistochemistry and sequencing in present study. A total of 106 cases, including 83 patients (78.3%) with mixed-type ACs, were assessed in the present study using eligible follow-up data. The ACs consisted of 32 acinar, 12 papillary, 5 mucinous, 11 micropapillary and 46 solid-predominant ACs. In total, 69.8% samples were composed of 2 or 3 histological components, with different expression levels of *CRKL* and *AXL*. ACs with *EGFR* mutation had a higher level of *AXL* expression compared with ACs without mutation (P=0.019). Multivariate survival analysis showed that AC subtypes and *EGFR* mutation subtypes were significantly associated with the progression-free survival (PFS) time. Acinar AC was the subtype with the most notable PFS time (30.6 months), which was significantly different from the PFS time of papillary, mucinous, micropapillary and solid-predominant ACs (hazard ratio, 0.4; 95% CI, 0.21-0.75; P=0.005). Among the ACs with exon 19 mutation, the median PFS time (28.8 months) of patients with a lower level of *AXL* protein expression was increased compared with the PFS time of patients with the L858R mutation and wild-type *EGFR* (9.1 months and 11 months, respectively; P=0.03), whereas no significant difference in ACs with an increased level of *AXL* expression. However, AC patients with higher level of *CRKL* expression had better PFS (28.8 months) than patients with the L858R mutation and wild-type *EGFR* (9.1 months and 11.3 months, respectively). Exon 19 deletion is an important status that is associated with an improved response to conventional chemotherapy. The identification of *EGFR* mutations combined with *CRKL* and *AXL* status may potentially alter the way that lung AC is treated.

Introduction

Adenocarcinoma (AC) is the most common histological type of non-small cell lung cancer (NSCLC) (1). Overall, >80% of lung ACs are diagnosed with mixed ACs, according to the 2004 World Health Organization (WHO) classification (2). Therefore, a semiquantitative evaluation system for calculating the components is necessary. A novel classification based on a multidisciplinary approach to the diagnosis of lung ACs was established by the International Association for the Study of Lung Cancer, American Thoracic Society and European Respiratory Society in 2011 (3). In the novel nomenclature system, invasive ACs are classified by predominant components, such as lepidic (formerly most mixed subtype tumors with non-mucinous BAC), mucinous (formerly mucinous BAC), acinar, papillary, solid patterns and micropapillary ACs (3). Although multidisciplinary data from widely divergent clinical, radiologic, molecular and pathological spectra accounts for the attributes of lung AC, it remains unclear how to address the biological properties of lung AC. Despite marked advances in the understanding of this tumor in the past decades, the production of universally accepted criteria for AC subtypes is required (4,5). Previous studies have reported that the new classification is an independent predictor of overall survival (6,7).

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Epidermal growth factor receptor (*EGFR*) mutations of exon 18 through exon 21 are reported to be associated with the sensitivity to tyrosine kinase inhibitors (TKIs); therefore, it is important to understand the nature of these mutations. *EGFR* mutations are mainly categorized into two groups, with 'classical' activating mutations including del19 and L858R. Additional analyses are required on other variants with unknown function (8). Tumor development in human lung ACs is increased by the activation of the *EGFR* signaling pathway. Therefore, target treatment with gefitinib leads to specific inhibition through apoptosis of cancer cells (9,10). A previous study on stage IB lung AC identified that micropapillary-predominant AC is the most common AC subtype with *EGFR* mutation, whereas solid-predominant AC has a lower frequency of *EGFR* mutation (11).

Echinoderm microtubule associated protein like 4 (*EML4*)-anaplastic lymphoma receptor tyrosine kinase (*ALK*), the major type of fusion gene resulting from *ALK* rearrangement, has been reported to be a potent oncogenic driver and a promising therapeutic target in ACs through the administration of crizotinib (12-14). Methods to detect *ALK* rearrangement include fluorescent *in situ* hybridization (FISH), immunohistochemistry (IHC) and reverse transcription-polymerase chain reaction (RT-PCR). FISH analysis is the only approved diagnostic test for the detection of the break-apart signal of *ALK* rearrangement. However, the disadvantages of the FISH test are that particular apparatuses are not always readily available in routine diagnostic laboratories, and subtle intrachromosomal rearrangement may be challenging to interpret; therefore, false-negative outcomes are inevitable. The subtle changes may be challenging to interpret by FISH analysis, and have led to false-negative results (15,16). IHC has been considered as an alternative to FISH for the detection of *ALK* rearrangement, and Ventana IHC for ALK fusion gene has been approved by the European Union (17).

Crk-like (*CRKL*) is upregulated in malignant tumors, including 49% of breast cancer, 55% of lung cancer, 67% of skin cancer, 50% of ovarian carcinoma, and 63% of colon carcinoma tumors (18). *CRKL* is a member of the human Crk adapter protein family and has been found to be amplified in lung cancer cells, with enhanced expression as a result of the amplification. In addition, knockdown of *CRKL* in lung cancer cell lines has led to a significant decrease in the proliferation, progression, survival, motility and invasiveness of lung cancer cells. All these data indicate that overexpression of CRKL may result in the oncogenic phenotype of lung cancer (19). Although evidence favors *CRKL* gene amplification in several human malignancies, including lung cancer, whether the expression of CRKL is associated with *EGFR* status in lung ACs remains to be elucidated.

AXL receptor tyrosine kinase (*AXL*) is confirmed to be associated with the carcinogenesis of numerous tumors. Elevated AXL expression and interaction with its ligand growth arrest-specific 6 (Gas6) have been associated with cell survival, proliferation, and migration in solid tumors (20,21). AXL is increasingly upregulated during the multistep process of esophageal carcinogenesis and is an adverse prognostic marker in esophageal AC (22). A previous study identified *AXL* activation as a novel mechanism of acquired resistance to *EGFR* inhibitors in non-small cell lung cancer (23). Overexpression of AXL is consistently manifested in prostate cancer cell lines and human prostate tumors. Blockage of AXL expression strongly inhibits the proliferation, migration and invasion of tumor cells, and therefore tumor growth (24).

The primary purpose of the present study is to analyze the correlation between the original expression levels of *CRKL* and *AXL* and status of the *ALK* and *EGFR* genes and the prognosis of different AC histological subtypes. Due to the presence of mixed AC components, it was important to evaluate the role of *CRKL* and *AXL* expression in AC subtypes combined with *EGFR* and *ALK* status.

Materials and methods

Study design. The present study is a retrospective review of 108 treatment-naive patients with AC, with samples consisting of 91 samples of resected primary lung cancer and 15 samples of metastatic nodules from advanced lung cancer from Beijing Chest Hospital (Beijing, China) between 2006 and 2012. All sample sections were evaluated by two pathologists to confirm the diagnosis and predominance (>70%) of tumor tissues. All slides were evaluated by pathologists based on the new classification (25) using a multi-headed microscope (objective, 40X; magnification, x400) and the clinical stage was defined according to the 7th Edition of the TNM Classification of the Union for International Cancer Control (3,26). A mean number of 4.5 slides (range, 2-11 slides) were reviewed. Patients were examined at 3-month intervals for the first 2 years following treatment and at 6-month intervals thereafter. The progression-free survival (PFS) time was measured from the date of treatment to the date of the first documented disease progression. The data were collected from the medical record system of Beijing Chest Hospital. The evaluation of disease progression included a physical examination, computed tomography scan of the chest and abdomen, brain magnetic resonance imaging, and bone scintigraphy. The last follow-up date was January 1, 2014. In total, 27 patients were censored from the current evaluation due to incomplete follow-up data.

DNA extraction, polymerase chain reaction amplification and direct sequencing for EGFR mutation. Genomic DNA was extracted from 50-100-mg tumor tissues obtained from formalin-fixed and paraffin-embedded blocks. The procedures followed a previously described protocol (27). PCR for exons 18-21 was performed using 100 ng template DNA in 50 μl volumes containing 0.75 U Hotstart Taq DNA polymerase (Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA), 5 μl PCR buffer, 0.8 μM dNTP (Fermentas; Thermo Fisher Scientific, Inc.), 0.5 μM of each primer (Sangon Biotech Co., Ltd., Shanghai, China), and various concentrations of $MgCl_2$, depending on varied markers. The nucleic acids used for the mutations were based on NM_005228.3. The primers were designed by Sangon Biotech Co., Ltd. as follows: Exon 18 forward, 5'-CAACCAAGCTCTCTTGAGGATC-3' and reverse, 5'-CCCAGCCCAGAGGCCTGT-3'; exon 19 forward, 5'-GCAGCATGTGGCACCATCTC-3' and reverse, 5'-AGAGCCATGGACCCCCACAC-3'; exon 20 forward, 5'-CACACTGACGTGCCTCTCC-3' and reverse, 5'-AGCAGGTACTGGGAGCCAAT-3'; and exon 21 forward, 5'-TCTGTCCCTCACAGCAGGGTCT-3' and reverse, 5'-GCTGGCTGACCT

AAAGCCACC-3'. The amplification and sequencing of exon fragments were performed as previously described (27). PCR products were sequenced in sense and antisense directions. Only specimens in which a mutation was identified in the two rounds were recorded as mutation-positive.

IHC for CRKL, AXL gene and ALK rearrangement. Tissue sections (4 μm) were prepared from tissue microarray blocks, deparaffinized using xylene and rehydrated through an ethanol series to water. Slides were incubated with the rabbit polyclonal anti-CRKL (catalog no., ab151791; Abcam Inc., Cambridge, UK) and polyclonal goat anti-AXL (catalog no., AF154; R&D Systems, Inc., Minneapolis, MN, USA) antibodies using a MaxVision horseradish peroxidase-polymer system kit (catalog nos. 5030 and 5108; Maixin Bio, Fuzhou, China). Incubation with the primary antibodies was performed overnight at 4˚C and at a 1:200 dilution. The MaxVision horseradish peroxidase-polymer system kit was used for immunostaining according to the manufacturer's instructions. Detection was accomplished using diaminobenzidine (DAB) (catalog no. CAS 7411-49-6; ImmunoCruz Staining System; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The slides were then counterstained in hematoxylin, and the stained tumor cells (≥1,000 cells), were scored by two independent observers. Cytoplasmic staining was considered positive for both CRKL and AXL. The immunoreactivity of carcinoma samples was semi-quantitatively evaluated by two aspects: Percentage of positive cells and staining. The staining strength was described as follows: 0, tumor cells were not stained; 1, light-yellow stained cells; 2, yellow stained cells; 3, brown stained cells. The observed area covered all histological patterns. The raw scores, ranging from 0 to 300, were calculated as follows: Percentage x staining strength. The final scores for statistical analysis were the average of raw scores from subtype components (Fig. 1A-D). Lower expression levels of AXL and CRKL were defined as a staining score ≤100, otherwise the tissues were classified as having a higher expression level.

IHC analysis for *ALK* rearrangement was completed by using Ventana method on a Benchmark XT autostainer (Roche Diagnostics, Indianapolis, IN, USA). This IHC method was an automatic staining by utilizing a ready-to-use primary anti-ALK rabbit monoclonal antibody (clone, D5F3; catalog no. 790-4794; Ventana Medical Systems, Inc., Tucson, AZ, USA). The staining procedure followed the Ventana ALK test protocol using an optiview amplification kit and an optiview DAB IHC detection kit. Presence or absence of *ALK* rearrangement was evaluated as positive or negative following the manufacturer's protocol (Roche Diagnostics). Neoplastic cells with diffuse dark brown cytoplasmic staining were classified as *ALK* rearrangement-positive; any other colors were classified as *ALK* rearrangement-negative (Fig. 1E).

Statistical analysis. *CRKL* and *AXL* expression was categorized into lower and higher levels of expression according to the aforementioned cut-off values. Association between *CRKL* and *AXL* expression were analyzed with clinicopathological factors by crosstab χ^2 test or Fisher's exact test (Table I). The impact of the following factors on the progression-free survival (PFS) rates was also evaluated: Gender; age; smoking status; clinical stage; *EGFR* gene status; and *ALK* fusion gene status. These clinicopathological factors were used in univariate and multivariate analyses to determine whether they had a significant effect on PFS (Table II). The survival rates and pairwise comparisons were stratified by clinicopathological characteristics and calculated using the Kaplan-Meier method and log-rank test. All statistical tests were two-sided and $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed on SAS system for Windows, version 9.2 (SAS Institute Inc., Cary, NC, USA). The survival curves were plotted using GraphPad Prism, version 6 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Patient characteristics and histopathological features. A total of 108 cases were originally involved in our study; however, the results of 2 cases were not incomplete in detecting the expression of AXL and CRKL. Therefore, 106 cases were analyzed in the present study, whose clinicopathological features are listed in Table I. Out of the 106 patients, AC was slightly more common in females (53.7%; 57/106). The median age was 55 years in females (range, 23-73 years) and 62 years in males (range, 33-79 years). All samples were diagnosed as invasive ACs and 82 (77.4%) of these samples were mixed ACs, which were classified into five histological subtypes based on their predominant components: 32 acinar ACs (30.19%), including 3 samples with cribriform pattern; 12 papillary ACs (11.32%); 5 mucinous carcinomas (4.72%); 11 micropapillary ACs (10.38%); and 46 solid ACs (43.40%). In total, 32.4% of patients did not have a smoking history. PFS comparisons among histological subtypes showed that acinar-predominant AC had a marginally improved chemotherapeutic response than others (P=0.052; Fig. 2A). Overall, 44.4% of patients were diagnosed at early stage (stage I and II), 39.8% of patients were diagnosed at stage IIIA, and 15.7% of patients were diagnosed at stage IIIB and IV. Conventional regimens of chemotherapy-combined use of carboplatin or cisplatin with Taxol®, gemcitabine, navelbine, docetaxel or pemetrexed were administered to 83 patients. Thymopeptide-5 alone was administered to 23 patients.

EGFR mutation and ALK rearrangement in lung ACs. Mutations of the *EGFR* kinase domain (exons 18-21) were successfully screened and 58 AC samples (53.7%) were positive for *EGFR* mutations (Table I), Two main types of *EGFR* mutation conferring TKI sensitivity are deletion in exon 19 and the missense mutation L858R in exon 21 (28). In the present study, 27 and 24 AC samples harbored deletion in exon 19 (range, K745-S753) and the L858R mutation, respectively. Deletion in exon 19 occurred in 12 acinar (12/27; 44.4%), 2 micropapillary (2/27; 7.4%), 3 papillary (3/27; 11.1%) and 10 solid (10/27; 37%) ACs. L858R mutation was found in 12 acinar ACs (12/24; 50%), consisting of one acinar with cribriform pattern, 1 micropapillary (1/24; 4.2%), 1 mucinous (1/24; 4.2%), 1 papillary (1/24; 4.2%) and 9 solid ACs (9/24; 37.5%). Deletion in exon 19 and L858R mutations were mainly harbored in acinar and solid-type ACs. Other less common mutations, such as G719A/S and S768I, were found in 6 patients.

Ventana IHC autostaining is a valid and convenient method for testing *ALK* fusion protein detection and the validity of

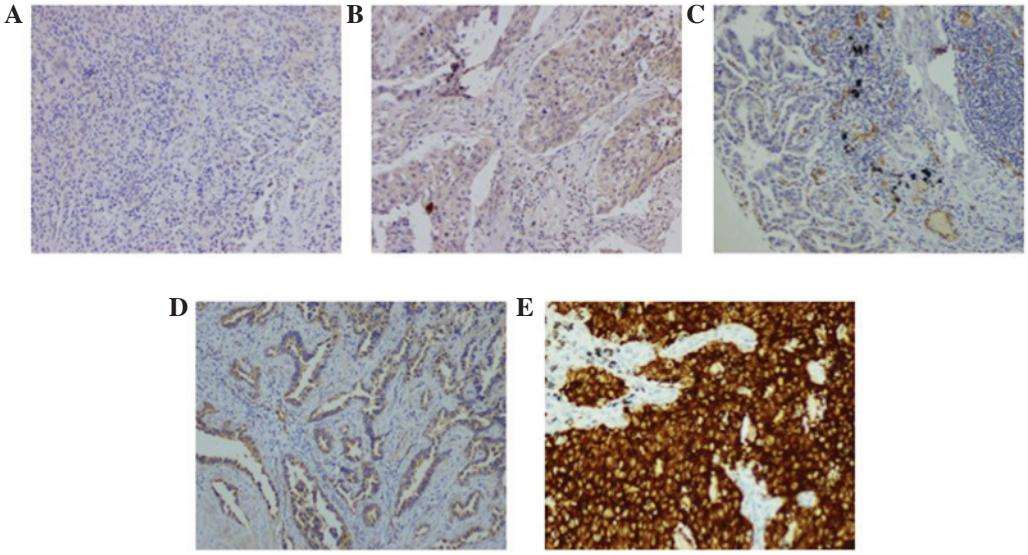

Figure 1. IHC staining of CRKL, AXL and ALK fusion protein in lung AC samples. (A and B) Representative acinar and solid predominant AC tissues. CRKL antibodies showed (A) lower expression level with weak cytoplasmic staining in acinar AC and (B) higher level of protein expression in a solid AC (original magnification, x100). (C and D) AXL immunostaining also showed (C) lower and (D) higher expression level, with weakly and moderately positive reaction in two acinar ACs, respectively (original magnification x100). (E) ALK rearrangement positive staining by Ventana IHC; brown staining granules full of cytoplasm could be observed (original magnification, x200). CRKL, Crk-like; AXL, AXL receptor tyrosine kinase; ALK, anaplastic lymphoma receptor tyrosine kinase; AC, adenocarcinoma; IHC, immunohistochemistry.

Ventana IHC has been verified by FISH (Fig. 1E) (29). In total, 17 ACs (17/106; 16.0%) were determined to be *ALK* fusion positive by Ventana IHC. In the current study, ACs with *ALK* rearrangement consisted of 2 micropapillary-predominant (2/11; 18.2%), 1 mucinous-predominant (1/5; 20.0%), 1 papillary-predominant (1/12; 8.3%) and 11 solid-predominant (11/46; 23.9%) tumors, particularly with cytoplasmic mucin. All these tissues stained positive for *ALK* rearrangement in a uniform pattern, despite the different morphological subtypes (Fig. 1E). In addition, 2/3 ACs with cribriform pattern were *ALK* rearrangement-positive. *EGFR* mutation and *ALK* rearrangement did not coexist in any AC subtype in the present study.

Effect of CRKL and AXL expression with different EGFR and ALK statuses on the prognosis of AC subtypes. Frequency crosstables were used to compare the staining intensity of *CRKL* and *AXL* with clinicopathological features (Table I; Fig. 1A-D). *CRKL* and *AXL* expression was not significantly associated with clinicopathological features, such as gender, AC subtypes, tumor size, *ALK* rearrangement and clinical stage (Table I). In total, 81 patients (81/106; 76.4%) experienced elapsed or progression during the follow-up period and 25 patients were censored, since they did not experience progressive disease or were lost to follow-up. The mean clinical follow-up period was 14.9 months (range, 1-48 months). In total, 50 and 58 patients were diagnosed at an early and advanced stage of disease, respectively. It was found that PFS time was not associated with chemotherapeutic regimens (P=0.17; Fig. 2B). However, the prognosis was significantly different between the two groups, with a median PFS time of 24.6 months (95% CI, 15.6-33.6 months) in the early-stage group and 10 months in the advanced group (95% CI, 5.8-14.1 months; HR, 2.83; 95% CI, 1.76-4.5; P<0.0001). The median PFS time of patients with acinar-predominant AC was 30.6 months, which was significantly longer than the micropapillary (HR, 2.9; 95% CI, 1.1-7.6; P=0.03), mucinous (HR, 2.73; 95% CI, 1.1-18.2; P=0.04), papillary (HR, 2.31; 95% CI, 1.1-7.3; P=0.016) and solid-predominant subtypes of AC (HR, 2.16; 95% CI, 1.2-3.5; P=0.009) (Table II). It is known that clinical staging is an important factor that affects the prognosis of patients with lung cancer. Subsequently, the PFS time of different subtypes were stratified by clinical staging and compared; it was found that only acinar AC had a longer PFS time than papillary ACs at an early stage of disease, but prognostic advantage was revealed at an advanced stage of disease (data not shown; P=0.025). The PFS time (16.5 months) of the 56 patients with *EGFR* mutation was increased compared with the PFS time of patients with wild-type *EGFR* (11 months; P=0.052). The mutation types were compared with the PFS of patients to elucidate the effect of *EGFR* mutation types on prognosis and found that they really played roles in prognosis of AC patients (P=0.01; Fig. 2C). It was also found that patients with exon 19 mutation (median PFS time, 27.8 months) had a strikingly improved PFS time compared with patients with L858R mutation (median PFS time, 10 months; HR, 2.5; 95% CI, 1.47-5.86; P=0.003) and wild-type (median PFS time, 11 months; HR, 2.2; 95% CI, 1.29-3.64; P=0.004) (Fig. 2C).

The median PFS time of 17 patients with the *ALK* fusion gene was 11 months (95% CI, 1.5-20.5 months), whereas the PFS time of the patients with wild-type *ALK* was 13 months (95% CI, 8.7-18.3 months). The prognosis of patients with wild-type *ALK* was better than the prognosis of patients with the *ALK* fusion gene (P=0.057).

No correlation was identified between *CRKL* and *AXL* expression and the clinical factors, such as gender, AC subtypes, tumor size, clinical stage, smoking history and *ALK* status. Despite this finding, the diverse staining resulted in the consideration of the components of ACs. Different staining patterns were correlated with the components of AC.

Table I. Association between *CRKL* and *AXL* expression and clinicopathological characteristics.

	CRKL expression, n (%)			*AXL* expression, n (%)		
Clinical	Lower level	Higher level	P-value	Lower level	Higher level	P-value
Gender			0.53			0.7
Male	16 (32.7)	33 (67.3)		21 (42.9)	28 (57.1)	
Female	15 (26.3)	42 (73.7)		27 (47.4)	30 (52.6)	
Adenocarcinoma subtypes			0.5			0.67
Acinar	13 (40.6)	19 (59.4)		12 (37.5)	20 (62.5)	
Micropapillary	3 (27.3)	8 (72.7)		4 (36.4)	7 (63.6)	
Papillary	2 (16.7)	10 (83.3)		6 (50.0)	6 (50.0)	
Solid	12 (26.1)	34 (73.9)		23 (50.0)	23 (50.0)	
Mucinous	1 (20.0)	4 (80.0)		3 (60.0)	2 (40.0)	
Tumor size			0.82			0.68
≤3 cm	9 (27.3)	24 (72.7)		16 (48.5)	17 (51.5)	
>3 cm	20 (30.3)	46 (69.7)		29 (43.9)	37 (56.1)	
EGFR status			0.83			0.019
Mutation	17 (30.4)	39 (69.6)		19 (33.9)	37 (66.1)	
Wild type	14 (28.0)	36 (72.0)		29 (58.0)	21 (42.0)	
ALK status			0.77			0.29
Fusion gene	4 (23.5)	13 (76.5)		10 (58.8)	7 (41.2)	
Non-fusion gene	27 (30.3)	62 (69.7)		38 (42.7)	51 (57.3)	
Smoking status			0.36			1.0
Never smoker	19 (26.0)	54 (74.0)		33 (45.2)	40 (54.8)	
Smoker or ever smoker	12 (36.4)	21 (63.6)		15 (45.5)	18 (54.5)	
Clinical stage			0.68			0.28
I+II	16 (32.7)	33 (67.3)		22 (44.9)	27 (55.1)	
IIIA	10 (24.4)	31 (75.6)		16 (39.0)	25 (61.0)	
IIIB+IV	5 (31.2)	11 (68.8)		10 (62.5)	6 (37.5)	

CRKL, Crk-like; *AXL*, AXL receptor tyrosine kinase; *EGFR*, epidermal growth factor receptor; *ALK*, anaplastic lymphoma receptor tyrosine kinase.

In total, 82 AC lesions (77.4%) were composed of ≥2 variant histological components, and the most frequent combination was solid and acinar patterns (26/106; 24.5%). Other mixtures consisted of solid, papillary, micropapillary and lepidic components of varied proportions. In addition, 46.2 and 24.5% ACs was observed with discrepant expression within components, respectively. In addition, 56 ACs (52.8%) with *EGFR* mutation had an increased level of *AXL* expression compared with ACs with wild-type *EGFR* (P=0.019). In total, 31 and 48 cases were determined with low expression of *CRKL* and *AXL*, respectively. A low level of *AXL* expression was detected in 48/106 ACs (45.3%) and exon 19 deletion was detected in 40.7% (11/27), L858R in 30.4% (7/23) and wild-type *EGFR* in 58% (29/50) of ACs. PFS comparison revealed that ACs with exon 19 deletion within the group with low *AXL* expression had a longer PFS time (median PFS time, 28.8 months) than those with the L858R mutation (median PFS time, 9.1 months; HR, 6.04; 95% CI, 6.15-117.7; P<0.0001) and wild-type *EGFR* (median PFS time, 11 months; HR, 2.88; 95% CI, 1.26-5.38; P=0.012) (Fig. 3A). There was no significant difference in PFS time among the AC subtypes with high *AXL* expression (P>0.05; Fig. 3B). Among the AC subtypes with low *CRKL* expression, the median PFS time of patients with exon 19 deletion (median PFS time, 19 months) was not significantly longer than the median PFS time of patients with the L858R mutation (median PFS time, 13 months) and wild-types *EGFR* (median PFS time, 9.97 months) (P=0.29; Fig. 3C). In contrast, for the 75 ACs with an increased level of *CRKL* expression, the 20 patients (20/75; 26.7%) with exon 19 deletion had a better PFS time (28.8 months) than the 14 patients (14/75; 18.7%) with the L858R mutation (9.1 months; HR, 2.79; 95% CI, 1.1-7.1; P=0.03) and all 50 patients (50/106; 47.2%) without *EGFR* mutation (11.3 months; HR, 2.49; 95% CI, 1.33-4.67; P=0.0046) (Fig. 3D).

Discussion

Overall, ~80% of lung ACs are categorized as mixed subtype according to the 2004 WHO classification (2). It has been proposed that a semiquantitative assessment of the percentages of various histological components, such as acinar, papillary, micropapillary, lepidic and solid, should be performed to

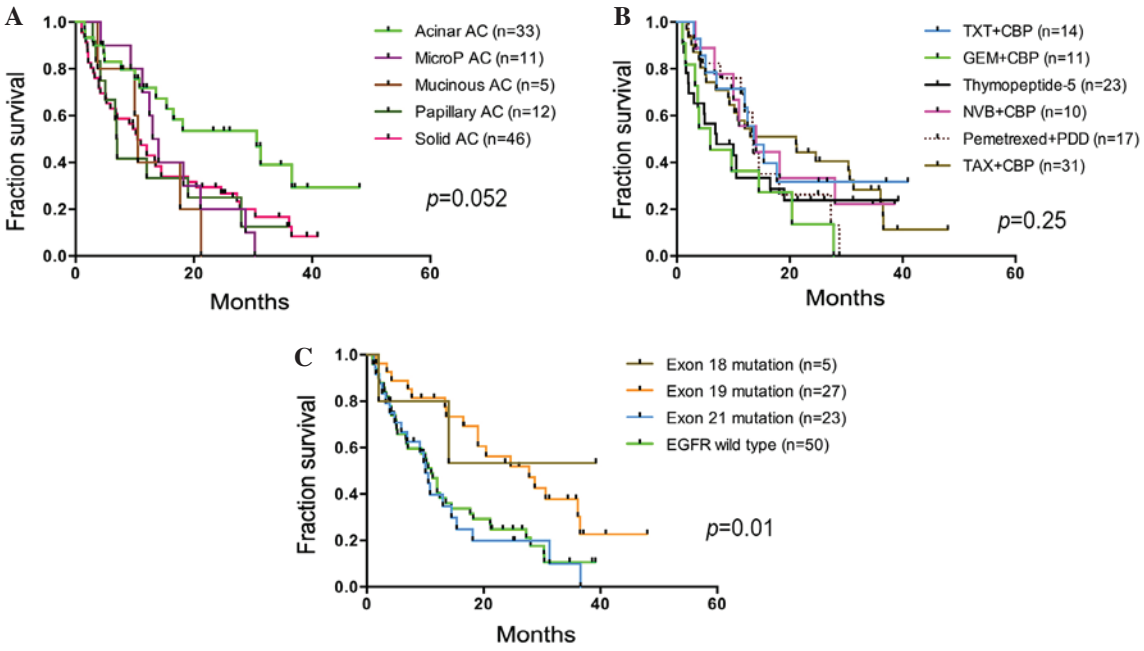

Figure 2. PFS comparisons among AC patients with different *EGFR* mutation types and treated with conventional chemotherapy. (A) PFS comparisons for patients having different histological subtypes. (B) PFS comparisons for patients receiving conventional chemotherapy. (C) PFS comparisons for patients harboring different *EGFR* status. *EGFR*, epidermal growth factor receptor; PFS, progression-free survival; AC, adenocarcinoma MicroP AC, micropapillary AC; TXT+CBP, docetaxel and carboplatin; GEM+CBP, gemcitabine and carboplatin; NVB+CBP, tavelbine and carboplatin; Pemetrexed+PDD, pemetrexed and cisplatin; TAX+CBP, Taxol® and carboplatin.

classify tumors according to the predominant components (30). It is crucial to adopt a practical way to address tumors that are comprised of a complex heterogeneous mixture of histological subtypes, since 70-90% of surgically resected lung tumors are diagnosed as invasive ACs (25). In previous years, multiple independent research groups have classified lung ACs according to the most predominant subtypes (30-34). Prominent diverse structures in morphology and heterogeneity in the biology of ACs have been considered in an increasing number of studies following the establishment of the new classification (3,35,36). The present study was commenced subsequent to a review of all the tissue sections, and the diagnoses were renewed based on the new classification. Studies on the topic of micropapillary AC have reported that patients in an early stage of disease have a poor prognosis (32,37). It has recently been shown that micropapillary tumors also have a poor prognosis, similar to that of ACs, with a predominantly solid subtype (38). Patients with papillary or acinar predominance or invasive mucinous AC show similar overall survival (OS). Patients with solid predominance and micropapillary predominance show the worst OS (6). In the present study, 106 AC patients were administrated with conventional chemotherapy, and the PFS times among different regimens were not significantly different (P>0.05). However, the prognosis of different AC histological mixtures was analyzed, and it was found that acinar-predominant AC had a longer PFS time (30.6 months) than other AC subtypes, despite the status of the *EGFR*, *ALK*, *CRKL* and *AXL* genes. A previous study reports that patients with micropapillary AC have a worse prognosis than patients with mucinous, solid and colloid AC (38). In the present cohort, the clinicopathological findings were analyzed by univariate Kaplan-Meier test and Cox regression analyses stratified with histological subtypes, *EGFR* and *ALK* status. The multivariate model showed that AC subtypes and *EGFR* mutation subtypes were independent factors that affected PFS time in addition to clinical stage. Thereafter, AC subtypes and *EGFR* status were compared with *AXL* and *CRKL* expression. Patients with ACs with exon 19 deletion (PFS time, 27.8 months) demonstrated an improved prognosis compared with patients with ACs with L858R mutation (PFS time, 10 months) and wild-type *EGFR* (PFS time, 11 months). A previous study investigating 44 patients with lung cancer has reported that the overall response rate to concurrent chemoradiotherapy is significantly increased in the *EGFR* mutant group compared with the wild-type *EGFR* group, and local regional relapse occurs less frequently in patients with *EGFR* mutation compared with patients with wild-type *EGFR* (39). It is well known that *EGFR* mutation is most common in ACs in the eastern Asian population, never-smokers and non-mucinous tumors (40-42). Lung cancer-associated *EGFR* mutations are clustered within the tyrosine kinase domain. In-frame exon 19 deletions occur just downstream of a lysine residue at position 745 (K745), which is critical for binding adenosine triphosphate. Absence of a few amino acids located C-terminal to this lysine residue may affect the configuration of the *EGFR* catalytic site (42). The L858R mutation occurs adjacent to the highly conserved DFG motif in the activation loop region of the kinase (43). Theoretically, these mutations may all result in conformational changes that lead to increased activity and TKI sensitivity (28,44). The actual mechanism of mutant *EGFR* tumors with target therapy has yet to be elucidated. The present results indicated that activating *EGFR* mutations, particularly in-frame deletions of exon 19, is more likely to be associated with clinical significance and it is necessary to consider the *EGFR* status of ACs. The *EGFR* status of ACs determined the response of the tumors to conventional chemotherapy.

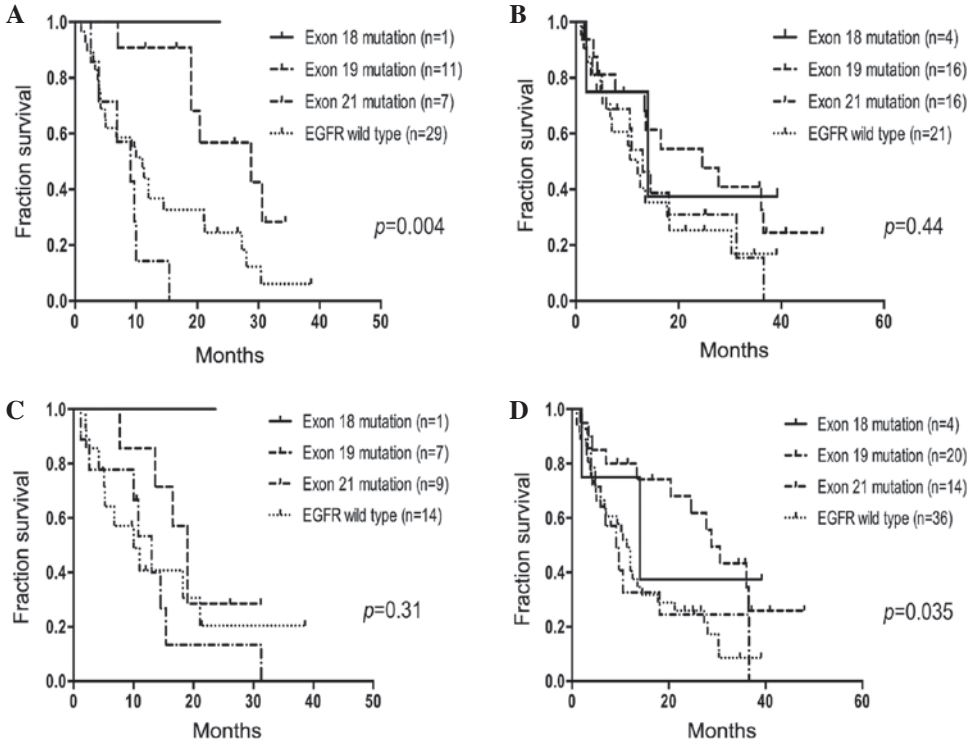

Figure 3. PFS comparisons among AC patients with different expression levels of CRKL and AXL protein combined with *EGFR* status. (A) PFS comparisons for patients with lower level of AXL expression and different *EGFR* mutation types. (B) PFS comparisons for patients with higher level of AXL expression and different *EGFR* mutation types. (C) PFS comparisons for patients with lower level of CRKL protein and different *EGFR* mutation types. (D) PFS comparisons for patients with higher level of CRKL protein and different *EGFR* mutation types. PFS, progression-free survival; AC, adenocarcinoma; CRKL, Crk-like; AXL, AXL receptor tyrosine kinase; *EGFR*, epidermal growth factor receptor.

ALK rearrangement results in fusion genes, such as *EML4-ALK*, *KIF5B-ALK* and *KLC1-ALK* (12,45,46). Detection of *ALK* rearrangement by FISH or RT-PCR is considered to be the standard procedure, but each method possesses limitations. The FISH method, which is based on a break-apart probe, has the limitation that it cannot determine the specific form of translocation, whereas RT-PCR cannot quantify the tumor cells with the *ALK* fusion gene. IHC using specific antibodies corresponds well with detecting the activating *ALK* fusion protein, and it may serve as a useful screening method with quantitation and quality outcome (15,16,46,47). The *ALK* fusion gene defines a distinct molecular subset of NSCLC, in particular AC, which benefits from treatment with *ALK*-inhibitors. Robust and reliable laboratory tests for predictive biomarkers are critical to select appropriate patients for targeted therapy. Patients with improved performance status and *EML4-ALK* translocation have an increased overall survival time compared with patients treated with conventional chemotherapy (17,48). There is no significant difference in clinical factors and survival outcome between the patients harboring variant 1 and those harboring non-variant 1 *EML4-ALK* fusion genes (49). The incidence of 17 ACs (16.0%) possessing *ALK* rearrangement in the present study was substantially more frequent than that in young male patients in Western countries (5.6%, 20/358) (15). No prognostic advantage of *ALK* translocation was demonstrated in the present study and the *ALK* fusion incidence was distributed regardless of histological predilection, which is consistent with other reports of *EML4-ALK* rearrangement (15,50,51). Improved prognosis was also identified in the patients without *ALK* rearrangement in the present study. We suspect that this discrepancy with other reports may be caused by the constituents resulting from the 'acinar', which is likely to lead to a better outcome than other histological subtypes. Additional stratified analysis is required to illuminate the prognostic difference of AC subtypes.

AXL is a transmembrane receptor tyrosine kinase whose overexpression has been reported in several human cancers. In addition, *Gas6-AXL* signaling promotes cell proliferation and survival, angiogenesis, and invasion and metastasis through activation of the *PI3K-AKT-mTOR* and *RAS-RAF-MEK-MAPK* pathways (52). *EGFR*-mutant lung cancer models *in vitro* and *in vivo* with increased activation of *AXL* have been shown to possess acquired resistance to erlotinib without T790 M alteration. In lung AC, *AXL* expression levels are associated with tumor advancement and the survival of patients with adjuvant chemotherapy, thus rendering *AXL* expression as a reliable biomarker and potential target for treatment of lung AC (53). In the present study, the role of *EGFR* activating mutation in the prognosis of AC patients was confirmed. Additional stratified pairwise comparisons were performed to elucidate the association between *AXL* and *EGFR* activating mutation types. An increased level of *AXL* expression was more evident in ACs with *EGFR* mutation. Investigation of the role of *AXL* in patient prognosis revealed an improved PFS time in patients with low expression of AXL and exon 19 mutation, compared to patients with higher expression of *AXL* and wild-type *EGFR*. The *AXL*/*Gas6* system remains an attractive therapeutic target (54), and certain small molecules with *AXL* inhibitory effects are already under development (55).

Table II. Association of PFS and hazard ratios with clinicopathological factors in ACs.

		Univariate analysis			Multivariate analysis		
Variables	Total, n	Median PFS, months	95% CI	P-value	Hazard ratio	95% CI	P-value
Gender							
Male	50	11.3	9.3-13.2	0.130			
Female	58	15.4	10.3-20.5				
Adenocarcinoma subtypes							
Acinar predominant	32	30.6	14.3-46.9	0.052	0.40	0.21-0.75	0.005[a]
MicroP predominant	11	13.0	10.6-15.4		0.61	0.28-1.32	0.210[a]
Mucinous predominant	5	10.5	9.4-11.6		0.96	0.37-2.52	0.940[a]
Papillary predominant	12	7.0	4.6-9.2		1.16	0.55-2.43	0.700[a]
Solid predominant	46	10.5	7.3-13.6				
CRKL expression score							
≤100	31	13.6	8.3-18.8	0.790	0.78	0.47-1.31	0.350
>100	75	12.5	9.0-15.9				
AXL expression score							
≤100	48	12.0	6.3-17.7	0.440	1.01	0.61-1.67	0.980
>100	58	13.5	11.2-15.8				
EGFR status[b]							
Mutation	56	16.5	11.4-21.6	0.053[c]			
Wild type	50	11.0	8.8-13.2				
Exon 18 mutation	5	14.0	11.4-23.7		0.69	0.16-2.96	0.610[d]
Exon 19 mutation	27	27.8	15.7-40.0		0.52	0.28-0.96	0.037[d]
Exon 21 mutation	23	10.0	7.8-12.2		1.33	0.70-2.55	0.390[d]
ALK status							
Fusion gene	17	11.0	1.5-20.5	0.057	1.45	0.79-2.64	0.230
Non-fusion gene	91	13.6	8.7-18.3				
Smoking status							
Never smoker	74	14.5	9.0-20.0	0.062			
Smoker or ever smoker	34	10.5	8.2-12.8				
Clinical stage (vs. I+II)[e]							
I+II	49	27.3	18.0-36.5	<0.001	1.97	1.38-2.83	<0.001
IIIA	42	10.0	5.2-14.7				
IIIB+IV	17	9.7	1.0-15.7				

[a]Comparison with solid predominant among AC subtypes. [b]Since the S768I mutation, which is located in exon 20, was found in one patient, this case was not listed in the table. Therefore, only 55 cases are listed in the table. [c]Comparison between *EGFR* mutation and *EGFR* wild type. [d]Comparison with *EGFR* wild type among *EGFR* mutation subtypes. [e]A total of 108 cases were originally involved in the present study, of which, the results of 2 cases were not incomplete in detecting the expression of AXL and CRKL. Therefore, these 2 cases were excluded from the study. PFS, progression-free survival; CI, confidence interval; CRKL, Crk-like; *AXL*, AXL receptor tyrosine kinase; *EGFR*, epidermal growth factor receptor; *ALK*, anaplastic lymphoma receptor tyrosine kinase.

CRKL expression is associated with enhanced cancer cell proliferation and invasion (18,19). Overexpression of *CRKL* is found in NSCLC and is associated with poor tumor differentiation, AC, advanced p-TNM stage, high proliferation index and poor overall survival. In addition, overexpression of *CRKL* in cell lines promotes cell proliferation by facilitating cell cycle progression (56). In the current study, the *CRKL* protein level was not demonstrated to be significantly associated with clinical features such as gender, smoking history, clinical staging, *EGFR* status and *ALK* status. However, overexpression or amplification of *CRKL* and activation of *AXL* is reported associated with the resistance to TKI (9,12,23,57). The original *CRKL* expression level in AC subtypes with various *EGFR* mutations remains limited; therefore, the comparison was performed in the present study to investigate the effect of *CRKL* expression concomitant with *EGFR* activated mutation on the prognosis of AC patients. Patients with high *CRKL* expression level and exon 19 mutation had an improved prognosis compared with other patients with a low *CRKL* expression level and other *EGFR* status. This indicates that overexpression of *CRKL* may confer an improved response to conventional chemotherapy in the ACs

with exon 19 mutation. To address the diverse expression of *AXL* and *CRKL*, the understanding of the exceptional histological and biological heterogeneity of lung carcinoma may be improved if the stem cell theory of cancer development is considered. According to these evolving views, neoplastic cells derive from abnormal progenitors that retain the potential to give rise to a diverse progeny, depending on the large variety of molecular abnormalities affecting the neoplastic clones. Therefore, neoplastic cells produce a large variety of carcinoma subtypes (12,58-63). The unique features of these abnormal precursors may determine the phenotype of neoplastic populations, organized in a hierarchy with various properties and degrees of differentiation (64). Numerous studies have focused on the pathological features of cases harboring *EGFR* mutations to evaluate the predictive significance of morphological characterization, but the available data are partly discordant (65,66). In a recent study in which the histological-genetic correlations of 100 ACs (94% mixed subtype) were analyzed, the strongest molecular correlation was observed between the *EGFR* mutation and the papillary subtype (30), as previously suggested (33). However, this contrasted with studies claiming that *EGFR* mutations mainly occur in the non-mucinous BAC and BAC AC mixed histological subtypes (67-69). In addition, previous studies investigating the morphological features of ACs that respond and do not respond to TKI therapies confirm that certain histological differences exist (70-72). However, there is morphological overlap, and WHO criteria may be considered a confounding factor. Following consideration of these limitations, the use of pure histology as a predictor for targeted *EGFR* inhibitory therapy in lung ACs is highly controversial. In accordance with the present results, the hypothesis that *EGFR* mutation does not have a predilection to particular histological subtypes was supported.

In conclusion, due to the incidence of *EGFR* mutation in the Asian population, it is necessary to consider the *EGFR* status as an underlying factor in limited therapeutic options. The present findings are considered to contribute to the understanding of *CRKL* and *AXL* expression as novel biomarkers and therapeutic targets in lung AC.

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